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(54) Title: METHODS FOR IMPROVING TRANSFORMATION EFFICIENCY

(57) Abstract

The present invention is drawn to compositions and methods for improving transformation efficiency. The compositions, synthetic marker genes, are used in transformation methods and result in increased transformation efficiency. The synthetic marker genes can be designed for maximum expression in any system.

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METHODS FOR IMPROVING TRANSFORMATION EFFICIENCY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-In-Part of Provisional application Serial No. 60/035,560 filed January 14, 1997.

FIELD OF THE INVENTION

The invention relates to the genetic modification of organisms, particularly plants.

BACKGROUND OF THE INVENTION

Gene transfer has offered great promise in the genetic manipulation of organisms. The movement of genes within plant species has played an important role in crop improvement for many decades. The recombinant DNA methods which have been developed have greatly extended the sources from which genetic information can be obtained for crop improvement. Gene transfer systems based on recombinant DNA are available for several crop species and are under development for many others.

Rapid progress has been made in developing the tools for manipulating genetic information in plants. Plant genes are being cloned, genetic regulatory signals deciphered, and genes transferred from entirely unrelated organisms to confer new agriculturally useful traits to crop plants. Recombinant DNA methods significantly increase the gene pool available for crop improvement.

A variety of methods have been developed for the transformation of plants and plant cells with DNA. Generally, the most success has been in dicotyledonous plants. Some success has been reported with certain monocotyledonous cereals.

Cereals comprise a commercially valuable group of plant species that could benefit from the introduction and expression of foreign genes controlling improved grain quality and such agronomically important traits as tolerance to disease, insects, herbicides, and stress. However, most cereals have not proven readily amenable to either *Agrobacterium*-mediated gene delivery, or to the routine regeneration of fertile transgenic plants from directly transformed protoplasts. The use of microprojectile-bombardment-mediated transformation of embryogenic tissue culture material, with the subsequent regeneration of transgenic plants, has overcome the regeneration problems associated with the production of plants from cereal protoplasts. Using this technology, transgenic plants have been obtained from microprojectile-bombarded tissue cultures of many species.

Many of the recent advances in plant science have resulted from application of the analytical power of recombinant DNA technology coupled with plant transformation. These approaches facilitate studies of the effects of specific gene alterations and additions on plant development and physiology. They also make possible the direct manipulation of genes to bio-engineer improved plant varieties.

While strides have been made in the genetic transformation of plants, it is by no means a routine matter. In fact, transformation efficiency is quite low making the process very labor intensive. Some reports indicate that the current transformation methods provide only a transformation frequency of about one event from every thousand bombarded embryos. This transformation frequency is too low for many genetic studies and for routine commerical applications. Therefore, a method is needed to improve the efficiency of genetic transformation.

SUMMARY OF THE INVENTION

Compositions and methods for improving transformation efficiency in organisms, particularly plants, are provided. The compositions, synthetic marker genes, are used in transformation methods and result in increased transformation efficiency. The synthetic marker genes can be designed for maximum expression in any system.

DESCRIPTION OF THE FIGURES

Figure 1: Nucleotide sequence of phosphinothricin acetyltransferase optimized for expression in monocots.

Figure 2: Nucleotide sequence of cyanamide hydratase optimized for expression in monocots.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is drawn to compositions and methods for improving transformation efficiency in organisms, particularly plants. For use in plants, the method involves stably transforming a plant cell or culture and regenerating plants from the transformed cells. Using the methods of the invention, fertile transgenic plants can be grown to maturity with a high frequency. The fertile transformed plants are capable of producing transformed progeny that express foreign genes of interest.

The methods of the present invention improve transformation efficiency. By improve efficiency it is intended that the number of transformed plants recovered by a transformation attempt is increased preferably at least two fold, preferably at least five fold, more preferably at least ten fold.

The present invention thus encompasses the fertile transgenic plants and transformed seeds thereof, as well as the subsequent progeny and products derived therefrom.

By transformation is intended the genetic manipulation of the plant, cell, cell line, callus, tissue, plant part, and the like. That is, such cell, cell line, callus, tissue, plant part, or plant which has been altered by the presence of recombinant DNA wherein said DNA is introduced into the genetic material within the cell, either chromosomally, or extra-chromosomally. Recombinant DNA includes foreign DNA, heterologous DNA, exogenous DNA, and chimeric DNA.

The transformed plants of the invention can be produced by genetic engineering. Alternatively, transformed parent plants can be produced by genetic

engineering and used to transfer the foreign genes into subsequent generations by sexual or asexual reproduction.

The methods of the present invention can be used in combination with any means for transformation of plants or plant cells. The present invention provides for the use of an optimized marker gene. The marker gene can be optimized for expression in a particular plant species, a particular genus of plants or a particular group of plants, for example monocots and/or dicots, maize, wheat, soybean, and the like.

By marker gene is intended both selectable marker genes and reporter genes. Both selectable marker genes and reporter genes facilitate identification and selection of transformed cells. To date, all genetic transformation systems which have been developed rely upon a selectable marker or reporter gene to enable the recovery of transgenic plants.

Reporter genes should ideally exhibit low background activity and should not have any detrimental effects on metabolism. The reporter gene products will have moderate stability *in vivo*, so that down-regulation of gene expression as well as gene activity can be detected. Finally, the reporter gene should be able to be assayed by a non-destructive, quantitative, sensitive, simple to perform and inexpensive system.

Reporter genes are known in the art and include but are not limited to:

Beta-glucuronidase (GUS) gene (Jefferson *et al.* (1991) *In Plant Molecular Biology Manual* (Gelvin *et al.*, eds.), pp. 1-33, Kluwer Academic Publishers). This gene is encoded by the uidA locus of *E. coli*. GUS enzyme activity can be assayed easily and sensitively in plants. The expression of GUS gene fusions can be quantified by fluorometric assay, and histochemical analysis can be used to localize gene activity in transgenic tissues.

Luciferase (DeWet *et al.* (1987) *Mol. Cell. Biol., 7*:725-737). Luciferase catalyzes the oxidation of D(-)-luciferin in the presence of ATP to generate oxyluciferin and yellow-green light.

Anthocyanins (Goff *et al.* (1990) *EMBO J., 9*:2517-2522). Anthocyanin is a reporter system that does not require the application of external substrates for its

detection. The anthocyanin system utilizes the C1, B and R genes, which code for trans-acting factors that regulate the anthocyanin biosynthetic pathway in maize seeds. The introduction of these regulatory genes under the control of constitutive promoters includes cell-autonomous pigmentation in non-seed tissues.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Kain *et al.* (1995) *BioTechniques, 19*:650-655 and Chiu *et al.* (1996) *Current Biology, 6*:325-330). GFP emits bright green light when excited with UV or blue light. GFP fluorescence does not require a substrate or cofactor, is stable, and can be monitored non-invasively in living cells.

Selectable marker genes are utilized for the selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (*NEO*) and hygromycin phosphotransferase (*HPT*) as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. (See DeBlock *et al.* (1987) *EMBO J., 6*:2513-2518; DeBlock *et al.* (1989) *Plant Physiol., 91*:691-704; Fromm *et al.* (1990) 8:833-839; Gordon-Kamm *et al.* (1990) 2:603-618) For example, resistance to glyphosate or sulfonylurea herbicides has been obtained by using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, bromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides.

For purposes of the present invention, selectable marker genes include, but are not limited to genes encoding: neomycin phosphotransferase II (Fraley *et al.* (1986) *CRC Critical Reviews in Plant Science, 4*:1-25); cyanamide hydratase (Maier-Greiner *et al.* (1991) *Proc. Natl. Acad. Sci. USA, 88*:4250-4264); aspartate kinase; dihydrodipicolinate synthase (Perl *et al.* (1993) *Bio/Technology, 11*:715-718); tryptophan decarboxylase (Goddijn *et al.* (1993) *Plant Mol. Bio., 22*:907-912); dihydrodipicolinate synthase and desensitized aspartade kinase (Perl *et al.*

(1993) *Bio/Technology, 11*:715-718); bar gene (Toki *et al.* (1992) *Plant Physiol., 100*:1503-1507 and Meagher *et al.* (1996) and *Crop Sci., 36*:1367); tryptophan decarboxylase (Goddijn *et al.* (1993) *Plant Mol. Biol., 22*:907-912); neomycin phosphotransferase (NEO) (Southern *et al.* (1982) *J. Mol. Appl. Gen., 1*:327; hygromycin phosphotransferase (HPT or HYG) (Shimizu *et al.* (1986) *Mol. Cell Biol., 6*:1074); dihydrofolate reductase (DHFR) (Kwok *et al.* (1986) *PNAS USA* 4552); phosphinothricin acetyltransferase (DeBlock *et al.* (1987) *EMBO J., 6*:2513); 2,2-dichloropropionic acid dehalogenase (Buchanan-Wollatron *et al.* (1989) *J. Cell. Biochem. 13D*:330); acetohydroxyacid synthase (Anderson *et al.*, U.S. Patent No. 4,761,373; Haughn *et al.* (1988) *Mol. Gen. Genet. 221*:266); 5-enolpyruvyl-shikimate-phosphate synthase (aroA) (Comai *et al.* (1985) *Nature 317*:741); haloarylnitrilase (Stalker *et al.*, published PCT applcn WO87/04181); acetyl-coenzyme A carboxylase (Parker *et al.* (1990) *Plant Physiol. 92*:1220); dihydropteroate synthase (sul I) (Guerineau *et al.* (1990) *Plant Mol. Biol. 15*:127); 32 kD photosystem II polypeptide (psbA) (Hirschberg *et al.* (1983) *Science, 222*:1346); etc.

Also included are genes encoding resistance to: chloramphenicol (Herrera-Estrella *et al.* (1983) *EMBO J., 2*:987-992); methotrexate (Herrera-Estrella *et al.* (1983) *Nature, 303*:209-213; Meijer *et al.* (1991) *Plant Mol Bio., 16*:807-820 (1991); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol., 5*:103-108; Zhijian *et al.* (1995) *Plant Science, 108*:219-227 and Meijer *et al.* (1991) *Plant Mol. Bio. 16*:807-820); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet., 210*:86-91); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res., 5*:131-137); bleomycin (Hille *et al.* (1986) *Plant Mol. Biol., 7*:171-176); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Bio., 15*:127-136); bromoxynil (Stalker *et al.* (1988) *Science, 242*:419-423); 2,4-D (Streber *et al.* (1989) *Bio/Technology, 7*:811-816); glyphosate (Shaw *et al.* (1986) *Science, 233*:478-481); phosphinothricin (DeBlock *et al.* (1987) *EMBO J., 6*:2513-2518).

See generally, G. T. Yarranton (1992) *Curr. Opin. Biotech., 3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA, 89*:6314-6318; Yao *et al.* (1992) *Cell, 71*:63-72; W. S. Reznikoff (1992) *Mol. Microbiol., 6*:2419-2422;

Barkley *et al.* (1980) *The Operon,* pp. 177-220; Hu *et al.* (1987) *Cell, 48*:555-566; Brown *et al.* (1987) *Cell, 49*:603-612; Figge *et al.* (1988) *Cell, 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA, 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA, 86*:2549-2553; Deuschle *et al.* (1990) *Science, 248*:480-483; M. Gossen (1993) PhD Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA, 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell Bio., 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA, 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA, 88*:5072-5076; Wyborski *et al.* (1991) *Nuc. Acids Res., 19*:4647-4653; A. Hillenand-Wissman (1989) *Topics in Mol. and Struc. Biol., 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother., 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry, 27*:1094-1104; Gatz *et al.* (1992) *Plant J., 2*:397-404; A. L. Bonin (1993) PhD Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA, 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother., 36*:913-919; Hlavka *et al.* (1985) *Handbook of Exp. Pharmacology, 78*; Gill *et al.* (1988) Nature 334:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker and reporter genes are not meant to be limiting. Any reporter or selectable marker gene are encompassed by the present invention. If necessary, such genes can be sequenced by methods known in the art.

The reporter and selectable marker genes are synthesized for optimal expression in the plant. That is, the coding sequence of the gene has been modified to enhance expression in plants. The synthetic marker gene is designed to be expressed in plants at a higher level resulting in higher transformation efficiency.

Methods for synthetic optimization of genes are available in the art. In fact, several genes have been optimized to increase expression of the gene product in plants. However, until the present invention no one had recognized that transformation efficiency could be improved by genetic modification of the marker gene for optimal expression in the cell being transformed.

The marker gene sequence can be optimized for expression in a particular plant species or alternatively can be modified for optimal expression in plant families. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest. See, for example, EPA 0359472; EPA 0385962; WO 91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA, 88*:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Research, 17*: 477-498. U.S. Patent No. 5,380,831; U.S. Patent No. 5,436,391; and the like, herein incorporated by reference. In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, fully optimized or partially optimized sequences may also be used.

In the same manner, genes can be optimized for expression in any organism. Thus, while the invention is described in relation to improving the transformation efficiency in plants, the methods of the invention can be applied to improving the transformation efficiency in any system.

The marker genes of the invention are provided in expression cassettes for expression in the organism of interest. In this manner, the cassette will include 5' and 3' regulatory sequences operably linked to the gene of interest. Additionally, the expression cassette may be linked at the 5' end to various promoters from the same or different organisms. These promoters would be selected for strength and/or inducibility. Examples of such promoters include but are not limited to the ubiquitin-1 (Ubi-1) promoter or the cauliflower mosaic virus 35S (CaMv) promoter. See for example Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689; Cornijo *et al.*. (1993) *Plant Mol. Biol.* 23:567-581; Hohn *et al.* (1993) *PNAS* 93(16):8334-8339. The expression cassette may also include 3' terminator regions linked to the gene of interest examples of which are the CaMV 35S terminator and the potato proteinase inhibitor protein or pin II terminator. See for example, Mitsuhara *et al.* (1996) *Plant Cell Physiol.* 37(1):49-59; Seymour *et al.* (1993) *Plant Mol. Biol.* 23(1):1-9; The cassette may additionally contain at least one gene to be cotransformed into the organism. Alternatively, the additional gene(s) of interest

can be provided on another expression cassette. Where appropriate, the additional gene(s) of interest may be optimized for increased expression in the transformed plant.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *PNAS USA, 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology, 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and P. Sarnow (1991) *Nature, 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987) *Nature, 325*:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. *et al.* (1989) *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.* (1991) *Virology, 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiology, 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resection, ligation, PCR, or the like may be employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

The compositions and methods of the present invention can be used in any transformation protocol. Such transformation protocols may vary depending on the type of plant or plant cell, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.*

(1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA, 83*:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology, 6*:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J., 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; WO91/10725 and McCabe *et al.* (1988) *Biotechnology, 6*:923-926). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet., 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology, 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674(soybean); McCabe *et al.* (1988) *Bio/Technology, 6*:923-926 (soybean); Datta *et al.* (1990) *Biotechnology, 8*:736-740(rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA, 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology, 6*:559-563 (maize); WO91/10725 (maize); Klein *et al.* (1988) *Plant Physiol., 91*:440-444(maize); Fromm *et al.* (1990) *Biotechnology, 8*:833-839; and Gordon-Kamm *et al.* (1990) *Plant Cell, 2*:603-618 (maize); Hooydaas-Van Slogteren & Hooykaas (1984) *Nature (London), 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA, 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) In *The Experimental Manipulation of Ovule Tissues,* ed. G.P. Chapman *et al.*, pp. 197-209. Longman, NY (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports, 9*:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet., 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell, 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports, 12*:250-255 and Christou and Ford (1995) *Annals of Botany, 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology, 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The plant plastid can also be transformed directly. Stable transformation of plastids have been reported in higher plants. See, for example, Svab *et al.* (1990) *Proc. Nat'l. Acad. Sci. USA, 87*:8526-8530; Svab & Maliga (1993) *Proc. Nat'l Acad. Sci. USA, 90*:913-917; Svab & Maliga (1993) *EMBO J., 12*:601-606. The method relies on particular gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by trans-activation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and

plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci., USA, 91*:7301-7305. Where the transformation protocol is directed to plastid transformation, the marker genes are optimized for expression in the plant plastid.

The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports, 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

While the present method has broad applicability, it is particularly useful in transforming plants which have been recalcitrant to known transformation methods. That is using the present method, maize elite lines, inbreds, and other lines difficult to transform can be transformed directly.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE I. GENERATION OF A MONOCOT-OPTIMIZED PAT GENE

The PAT gene, which confers resistance to the herbicide glufosinate ammonium, was originally cloned from *Streptomyces viridochromogenes*. The plasmid pB2/35SAcK consists of a synthetic plant-optimized PAT gene fused to a 35S-promoter and terminator cloned into pUC19. A second construct consisting of the PAT gene fused to a plant ubiquitin promoter was also utilized.

The PAT gene was first modified for expression in plants by replacing the GTG codon with ATG, and by modifying the four nucleotides upstream of the ATG codon to generate a plant-optimized PAT gene. The present invention relates to the further modification of the PAT gene to generate an example of a monocot-optimized selectable marker gene, the "monocot-optimized" PAT gene (moPAT).

Analysis of the success of monocot-optimization was determined by recovery of transformants when the monocot-optimized PAT gene was used as a selectable marker, resulting in the isolation of a large number of highly herbicide-resistant events.

In order to generate a monocot-optimized form of the PAT gene, the preferred codon usage patterns for maize were examined. See, for example, Adang, U.S. Patent No. 5,380,831. Information regarding the preferred codon usage of maize allowed for the replacement of codons with those codons that were more frequently used in maize. Codons were altered without altering the amino acid sequence of the PAT polypeptide. A codon usage table that reflects the codon usage of the monocot *Zea mays* was utilized to optimize the PAT gene expression in monocots, particularly maize. Because the codon usage among monocots is similar, the genes can be used in any monocot, for example, wheat. It is further recognized that monocot optimized sequences may express in acceptable levels in dicots.

The plant-optimized sequence of the PAT gene was translated and compared to the native protein sequence. The protein sequence was then back-translated to nucleotide sequence using the above-described maize codon usage table. Modifications of the nucleotide sequence were not made if such modification would result in alteration of the amino acid sequence of the encoded PAT protein. The basic methodology utilized to generate the monocot-optimized PAT gene sequence (moPAT) is outlined below:

a. The PAT protein amino acid sequence was "back-translated" to obtain a nucleotide sequence having those codons most frequently used in maize. A nucleotide sequence having codons reflecting preferred codon usage patterns of maize and encoding the PAT protein was determined. The amino acid sequence of the protein encoded by the back-translated, modified "monocot-optimized" nucleotide sequence was identical to the amino acid sequence encoded by the native PAT nucleotide sequence.

b. The nucleotide sequence was further modified by removal of regions of the gene including potential RNA processing sites, degradation sequences, and premature polyadenylation sequences.

c. Codons used <5% were avoided where possible.

d. The nucleotide sequence was further modified to delete clusters of A/T nucleotide pairs and G/C clusters having more than 10 G/C nucleotide pairs where possible. The GC content for maize genes is preferably 60-65% of the total nucleotide sequence.

e. Regions predicted to develop hairpin structures having a free energy of -12 kcal/mol were eliminated.

f. Cloning sites comprising a restriction enzyme recognition sequence may be added or removed.

g. Translation initiation sequence, based on a consensus from highly expressing maize genes (ACACGACACCatg), was added.

Other factors, such as those that influence transcriptional or translational initiation sizes, secondary structure of the gene or transcript, or result in modification of the poly(A) tail of the mRNA were additionally considered. A synthetic gene was then synthesized which incorporates such alterations and is shown in Figure 1.

EXAMPLE II. UTILIZATION OF THE moPAT GENE FOR INCREASED TRANSFORMATION EFFICIENCY

To confirm that the monocot-optimized gene was expressed in maize and produced a gene product in maize tissues, expression vectors containing the moPAT gene were transformed into maize cells or tissues. Several plasmids were generated in order to test the ability of the synthetic moPAT gene to function in a monocot-optimized fashion. Two separate methods were utilized in order to determine the efficiency and level of gene expression in monocots transformed with the monocot-optimized gene constructs. One method includes transformation of monocot cells with the monocot-optimized gene constructs followed by exposure of the cells to an herbicide (such as Bialaphos®, Basta, or glufosinate ammonium) to which the PAT

gene is known to confer resistance. The number of colonies recovered following exposure to the herbicide is an indication of the ability of the monocot-optimized gene to function in maize tissues and cells. If transformation is benefited, a greater number of transformed events (herbicide-resistant colonies) would be recovered following transformation with the moPAT gene than following transformation with the PAT gene.

The data indicated that transformation of cells (comprising model or elite maize genotypes) with the I8092 (Ubi::moPAT) plasmid followed by selection on Bialaphos resulted in the generation of resistant calli. Such resistant colonies appear at an earlier time point and grow at a faster rate than those cells transformed with the PAT gene construct I6609. Furthermore, the data indicated that transformation of maize cells with the I8092 plasmid comprising the moPAT gene resulted in the isolation of a greater number of transformed events than transformation with the I6609 plasmid comprising the PAT gene (Tables 1 and 2).

RESULTS OF TRANSFORMATIONS DONE WITH moPAT:

Table 1

Experiment	I6609 clones	I8092 (moPat) clones
1	12	59
2	8	56
3	7	1
4	6	6
5	5	3
6	0	1
7	0	2
Total:	**38**	**128**

Table 2

Experiment	Construct	# Plates Shot	# Resistant Calli	Frequency
1	6609	42	800	19.0%
2	6609	42	1006	23.4%
3	8092	36	1140	31.7%

4	8092	40	1116	27.9%

A second approach that was utilized to determine the ability of the moPAT gene to direct expression of the moPAT gene product in maize was detection of the PAT gene product in extracts of maize tissues. A large number of events transformed with I8092 (Ubi::moPAT) were regenerated. Table 3 gives the results of these experiments using monocots derived from transformation with either the I6609 plasmid comprising the PAT gene or the I8092 plasmid comprising the moPAT gene (ELISA values reported at pg/μg protein). The data indicates that the PAT gene product is detected at greater than 200 pg/μg total soluble protein (tsp) in a larger proportion of events transformed with the moPAT gene as compared to events transformed with the PAT gene. The data further indicated that transformation with the moPAT gene results in the recovery of a greater number of herbicide-resistant transformed events than recovery following transformation with the PAT gene.

moPAT ELISA RESULTS

Table 3

Construct	# Events Analyzed	Negative	1-50	51-100	>100
16609	46	20	12	6	8
18092	84	38	5	6	35

EXAMPLE III. UTILIZATION OF A MONOCOT-OPTIMIZED GENE FOR INCREASED RECOVERY OF EVENTS TRANSFORMED WITH A NON-SELECTABLE GENE OF INTEREST

There is a need in the art to produce large numbers of transgenic events when developing transgenic crops. As demonstrated in the above-described example (Example II), a monocot-optimized gene may be utilized to increase recovery of transformed events following selection with a drug to which the monocot-optimized gene confers resistance. An important obstacle encountered by

many skilled in the art is the inability to simply and accurately select for certain genes of interest. The moPAT gene functions in such a capacity, in that transformation of the moPAT gene with a gene of interest encoding a non-selectable gene product allows for selection of transformed events by selection of the transformed events in the present of a drug.

The gene encoding a nonselectable gene product may be encoded on the same plasmid comprising the moPAT gene or may be comprised within a separate plasmid or DNA molecule. If the gene of interest is provided on a separate plasmid, then it is likely, although not absolute, that the herbicide-resistant transformed events have been co-transfected with the non-selectable gene of interest, such as the cryIA(b) gene. Similarly, if the gene of interest resides on the same plasmid comprising the moPAT gene, then it is likely that herbicide-resistant events will comprise the gene of interest in addition to the moPAT gene. Therefore, by selection of transformed events using a drug to which the moPAT gene confers resistance, the probability of isolating a transformed event expressing the gene product of the gene of interest is increased. This is extremely important when producing transgenic crops in that large numbers of transgenic events must be isolated. Thus, the optimized gene enhances the ability to recover transformed events following transformation with a non-selectable gene of interest.

EXAMPLE IV. TRANSGENIC MAIZE COMPRISING A MONOCOT-OPTIMIZED moPAT GENE

To provide a maize plant comprising a monocot-optimized gene, a transgenic maize plant is generated by transformation of a monocot-optimized gene into a maize regenerable tissue followed by regeneration of said regenerable tissue into a mature trangenic maize plant. The maize regenerable tissue is transformed with an expression vector comprising a monocot-optimized gene. Following regeneration of the mature maize plant, tissues of the transgenic plant are harvested and assayed for the presence of the monocot-optimized gene.

Monocot cells were transformed by methods known in the art. See, for example, Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA, 85*:4305-4309 and Klein

et al. (1989) *Proc. Natl. Acad. Sci. USA, 86*:6681-6685, herein incorporated by reference. Generally, GS3 callus lines were the target issue. GS3 is a high-type II (model) corn genotype. Generally, the callus was sieved in perparation for bombardment.

After being bombed with particles coated with DNA at a concentration of about 0.1μg DNA/shot/plate, the tissue was maintained for two days on medium with no selection agent, afterwhich the tissue was transferred to medium with a selection agent (3μg/liter bialophos) to initiate the plant regeneration process.

Expression of the moPAT gene and its gene product confers a selective advantage to the transgenic plant. Thus, a transgenic plant is generated that has a selective advantage (herbicide resistance) over a non-transgenic plant.

EXAMPLE V. COMPARISON OF TRANSFORMATION EFFICIENCIES IN TRANSGENIC WHEAT (PAT vs moPAT).

PAT and moPAT were transformed into the cultivar Bobwhite (wheat) in order to determine whether the monocot-optimized CAH sequence would lead to improved transformation efficiencies in other plant species besides maize. Two experiments were conducted comparing the transformation efficiencies of the PAT gene with moPAT. Heads were harvested 12 - 14 days post anthesis, seeds sterilized in 20% sodium hypochlorite for 30 min., and rinsed three times in sterile water. Immature embryos were excised and plated on MS salts, 2% sucrose, 150 mg/l asparagine, 0.5 mg/l thiamine HC1, 1.5 mg/l 2,4-D, pH 5.8, solidified with 2.5 g/l Gelrite (initiation medium). Plates were incubated in the dark at 26°C. Embryos were transferred, five days post excision, to the above medium supplemented with 0.4M mannitol and cultured for four hours, then bombarded with 1 micron gold particles (0.083 ug DNA, 650 psi).

Twenty hours post-bombardment, embryos were transferred from the high osmotic medium to initiation medium containing 3 mg/l Bialaphos and cultured in the dark (16/8 photo-period). The embryos were subcultured approximately every 2 weeks for 4 months. Resistant calli were placed initially on regeneration media (MS salts and vitamins, 2% sucrose, 0.5 mg/l Dicamba, 3 mg/l Bialaphos, 2.5 mg/l Gelrite), and upon shoot formation, transferred to the same medium (minus

Dicamba) containing 5 mg/l Zeatin and transferred to light. Shoots were rooted in MS salts and vitamins at concentrations of 0.1 g/l myo-inositol, 4% sucrose, 0.7 mg/l IBA, 0.3 mg/l NAA, 1.5 g/l Gelrite. The transformation efficiency for the moPAT construct was triple that of PAT. The transformation efficiency for PAT was 0.3% and for moPAT was 0.9%, as is shown in Table 4.

PAT vs. moPAT TRANSFORMATION EFFICIENCY

Table 4

Gene	Dp#	# Embryos	# PCR+ Events	# PCR+ Events with seed	Trans. Efficiency
PAT	6609	625	4	2	0.3%
moPAT	8092	568	6	5	0.9%

EXAMPLE VI. GENERATION OF A MONOCOT-OPTIMIZED CAH SEQUENCE

Cyanamide in aqueous solution or in the form of its calcium salt is used as a fertilizer in agriculture. It also can act as an effective herbicide if applied prior to sowing. The enzyme cyanamide hydratase hydrates the nitrile group of cyanamide to form urea.

Cyanamide hydratase has been purified from *Myrothecium verrucaria*. See, Maier-Greiner *et al.* (1991) *Proc. Natl. Acad. Sci. USA, 88*:4260-4264, herein incorporated by reference. The gene encoding cyanamide hydratase was optimized for expression in maize by the methods outlines above. Codons were altered without altering the amino acid sequence of the enzyme. The nucleic acid and amino acid sequences of the optimized gene are given in Figure 2. .

EXAMPLE VII. ANALYSIS OF T1 PROGENY OF moCAH TRANSGENIC EVENTS:

To characterize the stability of the moCAH selectable marker from parent to progeny, maize cells were transformed with moCAH and successive generations were assayed for the presence of the moCAH gene. Three transgenic events were produced using the monocot-optimized cyanamide hydratase gene (moCAH) as the selectable marker. These events were confirmed at T0 plant level by Southern blot analysis. In order to confirm transgene inheritance in these events T1 plants were analyzed for the presence of the transgene using PCR and herbicide leaf painting techniques. Seeds were planted in soil in the greenhouse and plants were sampled at V4-5 leaf stage. In the first transgenic event thirty-nine T1 plants from one T0 plant were analyzed by PCR of which twenty five T1 plants were positive for the moCAH transgene. In the second transgenic event, two T0 plants were used. Fifty nine T1 plants from the first T0 were analyzed by PCR, of which thirty two were positive for the moCAH transgene. Out of fourteen T1 plants from the second T0 plant, eight T1 plants were positive for the moCAH transgene. None of the seventy-one T1seeds germinated in the third transgenic event due to poor seed quality and mold problems.

To find out whether the inherited moCAH gene was still functional, T1 plants at V5-6 stage were leaf-painted with a 10% solution of the commercial herbicide Dormex (containing 50% cyanamide). All control non-transformed plants were susceptible to the herbicide with the painted leaf showing severe damage. Some transgenic T1 plants from both transgenic events were completely resistant to 50% cyanamide demonstrating no damage from leaf painting. In Event #2, PCR analysis showing the presence of the moCAH transgene and leaf painting results demonstrating improved resistance to cyanamide displayed a segregation pattern of approximately 1:1. These results clearly demonstrate that the moCAH gene can be used efficiently as a selectable marker to transform maize plants and that the introduced moCAH gene can be stably integrated into the maize genome and transmitted to the following generation. The DNA construct that contained the moCAH gene was arranged on the integrating vector 10675 in the order Ubi

promoter;; moCAH gene;; PinII terminator. Sequences of the primers used for PCR confirmation of moCAH presence in the plant genome are given below.

primer 1: CTACAACCACTCCATGCGCGTGTTC

primer 2: CACATAACACACAACTTTGATGCCCAC

EXAMPLE VIII. COMPARISON OF TRANSFORMATION EFFICIENCIES IN TRANSGENIC WHEAT (CAH VS. moCAH).

To test whether the use of moCAH improved the transformation efficiency in other species, both CAH and moCAH were transformed into the wheat cultivar (Bobwhite). Four experiments were conducted to test CAH against moCAH transformation efficiency. Media used were as described above except that 37.5 mg/l cyanamide was substituted for Bialaphos.. No selection agent was used in the regeneration and rooting stages. The results showed a 0.0% transformation efficiency for CAH and a 0.8% transformation efficiency for moCAH.

CAH vs. moCAH TRANSFORMATION EFFICIENCY

Table 5

Gene	Dp#	# Embryos	# PCR+ Events	# PCR+ Events with seed	Trans. Efficiency
CAH	10660	652	0	0	0%
moCAH	10675	653	5	5	0.8%

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be

obvious that certain changes and modifications may be practiced within the scope of the appended claims.

IN THE CLAIMS

What is claimed is:

1. A method for transforming a plant, said method comprising ransforming a cell from said plant with a marker gene wherein said marker gene has been optimized for expression; and regenerating said cell into a transformed plant.

2. The method of claim 1, wherein said method comprises transforming said cell with at least on additional gene.

3. The method of claim 1, wherein said transformation is nuclear transformation.

4. The method of claim 1, wherein said transformation is chloroplast transformation.

5. The method of claim 1, wherein said plant is a monocot.

6. The method of claim 5, wherein said monocot is maize.

7. The method of claim 1, wherein said plant is a dicot.

8. The method of claim 1, wherein said marker gene is a selectable marker gene.

9. The method of claim 8, wherein said selectable marker gene is selected from a gene encoding antibiotic resistance or a gene encoding resistance to a herbicide.

10. The method of claim 9, wherein said gene encodes phosphinothricin acetyltransferase.

11. The method of claim 9, wherein said gene encodes cyanamide hydratase.

12. The method of claim 1, wherein said marker gene is a reporter gene.

13. The method of claim 12, wherein said reporter gene is green fluorescent protein.

14. A method for increasing transformation efficiency, said method comprising transforming a cell with a marker gene wherein said marker gene has been optimized for expression in said cell; and regenerating said transformed cells into a transformed organism.

15. The method of claim 14, wherein said organism is a plant.

16. The method of claim 15, wherein said plant is a monocot.

17. The method of claim 16, wherein said monocot is maize.

18. The method of claim 14, wherein said plant is a dicot.

19. The method of claim 12, wherein said transformation efficiency is increased at least two fold.

20. The method of claim 14, wherein said transformation efficiency is increased at least five fold.

21. The method of claim 14, wherein said transformation efficiency is increased at least ten fold.

22. The method of claim 14, wherein said method comprises transforming said cell with at least one additional gene.

23. A marker gene which has been modified to optimize expression in a plant.

24. The gene of claim 23, wherein said gene is a selectable marker gene.

25. The gene of claim 24, wherein said gene is selected from a gene encoding antibiotic resistance or a gene encoding resistance to a herbicide.

26. The gene of claim 25, wherein said gene encodes phosphinothricin acetyltransferase.

27. The gene of claim 26, wherein said gene encodes cyanamide hydratase.

28. The gene of claim 23, wherein said marker gene is a reporter gene.

29. The gene of claim 28, wherein said marker gene is green fluorescent protein.

30. A plant which has been transformed with the gene of claim 20.

31. Transformed seed from the plant of claim 30.

32. A plant which has been transformed with the gene of claim 27.

33. Transformed seed from the plant of claim 32.

34. A plant which has been transformed with the gene of claim 29.

35. Transformed seed from the plant of claim 34.

36. The gene of claim 26, wherein said gene has the nucleotide sequence given in Figure 1.

37. The gene of claim 27, wherein said gene has the nucleotide sequence given in Figure 2.

38. A plant having stably incorporated into its genome a marker gene, wherein said marker gene has been optimized for expression.

39. The plant of claim 38, wherein said plant comprises at least one additional chimeric gene incorporated into its genome.

40. Seed of the plant of claim 38.

41. Seed of the plant of claim 39.

FIGURE 1

MoPAT Sequence and translation:

```
  1 GGATCCACAC GACACCATGT CCCCCGAGCG CCGCCCCGTC GAGATCCGCC CGGCCACCGC
 61 CGCCGACATG GCCGCCGTGT GCGACATCGT GAACCACTAC ATCGAGACCT CCACCGTGAA
121 CTTCCGCACC GAGCCGCAGA CCCCGCAGGA GTGGATCGAC GACCTGGAGC GCCTCCAGGA
181 CCGCTACCCG TGGCTCGTGG CCGAGGTGGA GGGCGTGGTG GCCGGCATCG CCTACGCCGG
241 CCCGTGGAAG GCCCGCAACG CCTACGACTG GACCGTGGAG TCCACCGTGT ACGTGTCCCA
301 CCGCCACCAG CGCCTCGGCC TCGGCTCCAC CCTCTACACC CACCTCCTCA AGAGCATGGA
361 GGCCCAGGGC TTCAAGTCCG TGGTGGCCGT GATCGGCCTC CCGAACGACC CGTCCGTGCG
421 CCTCCACGAG GCCCTCGGCT ACACCGCCCG CGGCACCCTC CGCGCCGCCG GCTACAAGCA
481 CGGCGGCTGG CACGACGTCG GCTTCTGGCA GCGCGACTTC GAGCTGCCGG CCCCGCCGCG
541 CCCGGTGCGC CCGGTGACGC AGATCTGAGG TGTCGTGTTA AC

   +1 MetSerProGluArgArgProValGluIleArgProAlaThrAlaAlaAspMetAlaAla
    1 ATGTCCCCCGAGCGCCGCCCCGTCGAGATCCGCCCGGCCACCGCCGCCGACATGGCCGCC
      TACAGGGGGCTCGCGGCGGGGCAGCTCTAGGCGGGCCGGTGGCGGCGGCTGTACCGGCGG

   +1 ValCysAspIleValAsnHisTyrIleGluThrSerThrValAsnPheArgThrGluPro
   61 GTGTGCGACATCGTGAACCACTACATCGAGACCTCCACCGTGAACTTCCGCACCGAGCCG
      CACACGCTGTAGCACTTGGTGATGTAGCTCTGGAGGTGGCACTTGAAGGCGTGGCTCGGC

   +1 GlnThrProGlnGluTrpIleAspAspLeuGluArgLeuGlnAspArgTyrProTrpLeu
  121 CAGACCCCGCAGGAGTGGATCGACGACCTGGAGCGCCTCCAGGACCGCTACCCGTGGCTC
      GTCTGGGGCGTCCTCACCTAGCTGCTGGACCTCGCGGAGGTCCTGGCGATGGGCACCGAG

   +1 ValAlaGluValGluGlyValValAlaGlyIleAlaTyrAlaGlyProTrpLysAlaArg
  181 GTGGCCGAGGTGGAGGGCGTGGTGGCCGGCATCGCCTACGCCGGCCCGTGGAAGGCCCGC
      CACCGGCTCCACCTCCCGCACCACCGGCCGTAGCGGATGCGGCCGGGCACCTTCCGGGCG

   +1 AsnAlaTyrAspTrpThrValGluSerThrValTyrValSerHisArgHisGlnArgLeu
  241 AACGCCTACGACTGGACCGTGGAGTCCACCGTGTACGTGTCCCACCGCCACCAGCGCCTC
      TTGCGGATGCTGACCTGGCACCTCAGGTGGCACATGCACAGGGTGGCGGTGGTCGCGGAG

   +1 GlyLeuGlySerThrLeuTyrThrHisLeuLeuLysSerMetGluAlaGlnGlyPheLys
  301 GGCCTCGGCTCCACCCTCTACACCCACCTCCTCAAGAGCATGGAGGCCCAGGGCTTCAAG
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FIGURE 1 (cont.)

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     CCGGAGCCGAGGTGGGAGATGTGGGTGGAGGAGTTCTCGTACCTCCGGGTCCCGAAGTTC

  +1 SerValValAlaValIleGlyLeuProAsnAspProSerValArgLeuHisGluAlaLeu
361  TCCGTGGTGGCCGTGATCGGCCTCCCGAACGACCCGTCCGTGCGCCTCCACGAGGCCCTC
     AGGCACCACCGGCACTAGCCGGAGGGCTTGCTGGGCAGGCACGCGGAGGTGCTCCGGGAG

  +1 GlyTyrThrAlaArgGlyThrLeuArgAlaAlaGlyTyrLysHisGlyGlyTrpHisAsp
421  GGCTACACCGCCCGCGGCACCCTCCGCGCCGCCGGCTACAAGCACGGCGGCTGGCACGAC
     CCGATGTGGCGGGCGCCGTGGGAGGCGCGGCGGCCGATGTTCGTGCCGCCGACCGTGCTG

  +1 ValGlyPheTrpGlnArgAspPheGluLeuProAlaProProArgProValArgProVal
481  GTCGGCTTCTGGCAGCGCGACTTCGAGCTGCCGGCCCCGCCGCGCCCGGTGCGCCCGGTG
     CAGCCGAAGACCGTCGCGCTGAAGCTCGACGGCCGGGGCGGCGCGGGCCACGCGGGCCAC

  +1 ThrGlnIle***
541  ACGCAGATCTGA
     TGCGTCTAGACT
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FIGURE 2

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   1 TCGCGCGTTT  CGGTGATGAC  GGTGAAAACC  TCTGACACAT  GCAGCTCCCG
     AGCGCGCAAA  GCCACTACTG  CCACTTTTGG  AGACTGTGTA  CGTCGAGGGC

  51 GAGACGGTCA  CAGCTTGTCT  GTAAGCGGAT  GCCGGGAGCA  GACAAGCCCG
     CTCTGCCAGT  GTCGAACAGA  CATTCGCCTA  CGGCCCTCGT  CTGTTCGGGC

 101 TCAGGGCGCG  TCAGCGGGTG  TTGGCGGGTG  TCGGGGCTGG  CTTAACTATG
     AGTCCCGCGC  AGTCGCCCAC  AACCGCCCAC  AGCCCCGACC  GAATTGATAC

 151 CGGCATCAGA  GCAGATTGTA  CTGAGAGTGC  ACCATATGCG  GTGTGAAATA
     GCCGTAGTCT  CGTCTAACAT  GACTCTCACG  TGGTATACGC  CACACTTTAT

 201 CCGCACAGAT  GCGTAAGGAG  AAAATACCGC  ATCAGGCGCC  ATTCGCCATT
     GGCGTGTCTA  CGCATTCCTC  TTTTATGGCG  TAGTCCGCGG  TAAGCGGTAA

 251 CAGGCTGCGC  AACTGTTGGG  AAGGGCGATC  GGTGCGGGCC  TCTTCGCTAT
     GTCCGACGCG  TTGACAACCC  TTCCCGCTAG  CCACGCCCGG  AGAAGCGATA

 301 TACGCCAGCT  GGCGAAAGGG  GGATGTGCTG  CAAGGCGATT  AAGTTGGGTA
     ATGCGGTCGA  CCGCTTTCCC  CCTACACGAC  GTTCCGCTAA  TTCAACCCAT
                                                    HindIII

 351 ACGCCAGGGT  TTTCCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGCCAA
     TGCGGTGCCA  AAAGGGTCAG  TGCTGCAACA  TTTTGCTGCC  GGTCACGGTT
     HindIII                            HpaI
 401 GCTTGCATGC  CTGCAGGTCG  ACTCTAGAGT  TAACACGACA  CCTCACTCCC
     CGAACGTACG  GACGTCCAGC  TGAGATCTCA  ATTGTGCTGT  GGAGTGAGGG

 451 ACGGCTTCAT  CAGGGTGTTG  GCCTCCATCT  GCTTGTCGAA  CTGCGGGATG
     TGCCGAAGTA  GTCCCACAAC  CGGAGGTAGA  CGAACAGCTT  GACGCCCTAC

 501 TGGGTGGTGT  GGCACCACGG  CTTGTTGGAC  TCCTCCTTGC  GCACGGTGCA
     ACCCACCACA  CCGTGGTGCC  GAACAACCTG  AGGAGGAACG  CGTGCCACGT

 551 GGCGAACCAG  GAGCACCAGC  CGTGGCGCGG  GAAGGCGGTG  TTGATGGAGT
     CCGCTTGGTC  CTCGTGGTCG  GCACCGCGCC  CTTCCGCCAC  AACTACCTCA

 601 TGCGGGTGGT  GTCGTCCACC  CAGGAGCCGA  AGTCGTCGAT  GCCGTCGTAG
     ACGCCCACCA  CAGCAGGTGG  GTCCTCGGCT  TCAGCAGCTA  CGGCAGCATC

 651 GCGCCCACGT  TGTCGTAGAG  GGTGGCGAGC  TGGATGAGCT  GGCCGAGGAA
     CGCGGGTGCA  ACAGCATCTC  CCACCGCTCG  ACCTACTCGA  CCGGCTCCTT

 701 GGTGATGTTG  CCGTCGACGC  CGACGTCCTC  GTGGCGGATG  ATGGCCTCGG
     CCACTACAAC  GGCAGCTGCG  GCTGCAGGAG  CACCGCCTAC  TACCGGAGCC

 751 CCACCGCCTC  CGCCTGGTCG  GTGGAGGAGC  CGAGCACCT   GAGGACCTCC
     GGTGGCGGAG  GCGGACCAGC  CACCTCCTCG  GCTCGTGGAA  CTCCTGGAGG

 801 ATCGCCTTGA  TGCCGCCGTA  GATGTCGAAG  GACATGCGGG  TGGAGGTGAA
     TAGCGGAACT  ACGGCGGCAT  CTACAGCTTC  CTGTACGCCC  ACCTCCACTT

 851 GTAGGCCTCG  GCGGTGCCCA  CGTCGTGGAG  GAGGCAGGTG  AGGGCCCAGG
     CATCCGGAGC  CGCCACGGGT  GCAGCACCTC  CTCCGTCCAC  TCCCGGGTCC
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FIGURE 2 (cont.)

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 901  TGGACGGGGA GAGGTCCTTG GCCTGCTCCG GGAGGAGCCT CCTGGCGATC
      ACCTGCCCCT CTCCAGGAAC CGGACGAGGC CCTCCTCGGA GGACCGCTAG

 951  ACGGTGCCCC AGTAGAACAC GCGCATGGAG TGGTTGTAGG TCTCCGGGGA
      TGCCACGGGG TCATCTTGTG CGCGTACCTC ACCAACATCC AGAGGCCCCT

1001  GAGGCGGGCC TTGACGAACG CCTGGGCCTC GGCCACGAGC TTGTCGGCCG
      CTCCGCCCGG AACTGCTTGC GGACCCGGAG CCGGTGCTCG AACAGCCGGC

1051  CTGGGAAGGC GATGTCCTCC ACGGAGTAGG AGCTGACGTC GCCGAGCTTG
      GACCCTTCCG CTACAGGAGG TGCCTCATCC TCGACTGCAG CGGCTCGAAC

1101  CCGAGGGAGT CCACGATGGC CTTGGCGCTG ACGGGGACCG CGGTCCAGCC
      GGCTCCCTCA GGTGCTACCG GAACCGCGAC TGCCCCTGGC GCCAGGTCGG

                                                  BamHI
1151  GTTGGCCTTC ACCTCGGAGG ACGACATGGT GTCGTGTGGA TCCCCGGGTA
      CAACCGGAAG TGGAGCCTCC TGCTGTACCA CAGCACACCT AGGGGCCCAT
        EcoRI
1201  CCGAATTCGT AATCATGGTC ATAGCTGTTT CCTGTGTGAA ATTGTTATCC
      GGCTTAAGCA TTAGTACCAG TATCGACAAA GGACACACTT TAACAATAGG

1251  GCTCACAATT CCACACAACA TACGAGCCGG AAGCATAAAG TGTAAAGCCT
      CGAGTGTTAA GGTGTGTTGT ATGCTCGGCC TTCGTATTTC ACATTTCGGA

1301  GGGGTGCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT GCGCTCACTG
      CCCCACGGAT TAGTCACTCG ATTGAGTGTA ATTAACGCAA CGCGAGTGAC

1351  CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCGG
      GGGCGAAAGG TCAGCCCTTT GGACAGCACG GTCGACGTAA TTACTTAGCC

1401  CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT
      GGTTGCGCGC CCCTCTCCGC CAAACGCATA ACCCGCGAGA AGGCGAAGGA

1451  CGCTCACTGA CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG AGCGGTATCA
      GCGAGTGACT GAGCGACGCG AGCCAGCAAG CCGACGCCGC TCGCCATAGT

1501  GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG GGGATAACGC
      CGAGTGAGTT TCCGCCATTA TGCCAATAGG TGTCTTAGTC CCCTATTGCG

1551  AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG AACCGTAAAA
      TCCTTTCTTG TACACTCGTT TTCCGGTCGT TTTCCGGTCC TTGGCATTTT

1601  AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC TGACGAGCAT
      TCCGGCGCAA CGACCGCAAA AAGGTATCCG AGGCGGGGGG ACTGCTCGTA

1651  CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA
      GTGTTTTTAG CTGCGAGTTC AGTCTCCACC GCTTTGGGCT GTCCTGATAT

1701  AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC
      TTCTATGGTC CGCAAAGGGG GACCTTCGAG GGAGCACGCG AGAGGACAAG

1751  CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC
      GCTGGGACGG CGAATGGCCT ATGGACAGGC GGAAAGAGGG AAGCCCTTCG
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FIGURE 2 (cont.)

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1801  GTGGCGCTTT CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT
      CACCGCGAAA GAGTATCGAG TGCGACATCC ATAGAGTCAA GCCACATCCA

1851  CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC
      GCAAGCGAGG TTCGACCCGA CACACGTGCT TGGGGGGCAA GTCGGGCTGG

1901  GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAGACAC
      CGACGCGGAA TAGGCCATTG ATAGCAGAAC TCAGGTTGGG CCATTCTGTG
                          AlwNI
1951  GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG
      CTGAATAGCG GTGACCGTCG TCGGTGACCA TTGTCCTAAT CGTCTCGCTC

2001  GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT
      CATACATCCG CCACGATGTC TCAAGAACTT CACCACCGGA TTGATGCCGA

2051  ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC
      TGTGATCTTC CTGTCATAAA CCATAGACGC GAGACGACTT CGGTCAATGG

2101  TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAACAAA CCACCGCTGG
      AAGCCTTTTT CTCAACCATC GAGAACTAGG CCGTTTGTTT GGTGGCGACC

2151  TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAAG
      ATCGCCACCA AAAAAACAAA CGTTCGTCGT CTAATGCGCG TCTTTTTTTC

2201  GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG
      CTAGAGTTCT TCTAGGAAAC TAGAAAAGAT GCCCCAGACT GCGAGTCACC

2251  AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT
      TTGCTTTTGA GTGCAATTCC CTAAAACCAG TACTCTAATA GTTTTTCCTA

2301  CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA
      GAAGTGGATC TAGGAAAATT TAATTTTTAC TTCAAAATT  AGTTAGATTT

2351  GTATATATGA GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG
      CATATATACT CATTTGAACC AGACTGTCAA TGGTTACGAA TTAGTCACTC

2401  GCACCTATCT CAGCGATCTG TCTATTTCGT TCATCCATAG TTGCCTGACT
      CGTGGATAGA GTCGCTAGAC AGATAAAGCA AGTAGGTATC AACGGACTGA

2451  CCCCGTCGTG TAGATAACTA CGATACCGGA GGGCTTACCA TCTGGCCCCA
      GGGGCAGCAC ATCTATTGAT GCTATGCCCT CCCGAATGGT AGACCGGGGT

2501  GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA
      CACGACGTTA CTATGGCGCT CTGGGTGCGA GTGGCCGAGG TCTAAATAGT

2551  GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC
      CGTTATTTGG TCGGTCGGCC TTCCCGGCTC GCGTCTTCAC CAGGACGTTG

2601  TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA
      AAATAGGGGG AGGTAGGTCA GATAATTAAC AACGGCCCTT CGATCTCATT

2651  GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTACAGGC
      CATCAAGCGG TCAATTATCA AACGCGTTGC AACAACGGTA ACGATGTCCG
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FIGURE 2 (cont.)

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2701 ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC
     TAGCACCACA GTGCGAGCAG CAAACCATAC CGAAGTAAGT CGAGGCCAAG

2751 CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG
     GGTTGCTAGT TCCGCTCAAT GTACTAGGGG GTACAACACG TTTTTTCGCC

2801 TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG
     AATCGAGGAA GCCAGGAGGC TAGCAACAGT CTTCATTCAA CCGGCGTCAC

2851 TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC
     AATAGTGAGT ACCAATACCG TCGTGACGTA TTAAGAGAAT GACAGTACGG

2901 ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT
     TAGGCATTCT ACGAAAAGAC ACTGACCACT CATGAGTTGG TTCAGTAAGA

2951 GAGAATAGTG TATGCGGCCA CCGAGTTGCT CTTGCCCGGC GTCAATACGG
     CTCTTATCAC ATACGCCGCT GGCTCAACGA GAACGGGCCG CAGTTATGCC
                                                 Asp700
3001 GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA TCATTGGAAA
     CTATTATGGC GCGGTGTATC GTCTTGAAAT TTTCACGAGT AGTAACCTTT
     Asp700
3051 ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA
     TGCAAGAAGC CCCGCTTTTG AGAGTTCCTA GAATGGCGAC AACTCTAGGT

3101 GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT
     CAAGCTACAT TGGGTGAGCA CGTGGGTTGA CTAGAAGTCG TAGAAAATGA

3151 TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA
     AAGTGGTCGC AAAGACCCAC TCGTTTTTGT CCTTCCGTTT TACGGCGTTT

3201 AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT
     TTTCCCTTAT TCCCGCTGTG CCTTTACAAC TTATGAGTAT GAGAAGGAAA

3251 TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC
     AAGTTATAAT AACTTCGTAA ATAGTCCCAA TAACAGAGTA CTCGCCTATG

3301 ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC CGCGCACATT
     TATAAACTTA CATAAATCTT TTTATTTGTT TATCCCCAAG GCGCGTGTAA

3351 TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT ATCATGACAT
     AGGGGCTTTT CACGGTGGAC TGCAGATTCT TTGGTAATAA TAGTACTGTA

3401 TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTC
     ATTGGATATT TTTATCCGCA TAGTGCTCCG GGAAAGCAG
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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/00359

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/54 C12N15/82 A01H5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RATHORE K. ET AL.: "Use of bar as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts" PLANT MOLECULAR BIOLOGY, vol. 21, no. 5, March 1993, pages 871-884, XP002064546 see the whole document ---	1-3,5, 8-10, 23-26, 30,31, 38-41
X	TIAN L. ET AL.: "High level of expression of modified green fluorescent protein gene transfer in conifer tissues" IN VITRO, vol. 32, no. 4, October 1996 - December 1996, page 311 XP002064547 see the whole document ---	23,24, 28,29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1998	27/05/1998

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INTERNATIONAL SEARCH REPORT

Interi ...nal Application No
PCT/US 98/00359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	WO 96 27675 A (MEDICAL RES COUNCIL ;HASELOFF JAMES PHILLIP (AU); HODGE SARAH (GB)) 12 September 1996 * see esp. pp. 3-8 *	1-3,7,8, 12,13, 23,24, 28,29
X	CHIU W -L ET AL: "ENGINEERED GFP AS A VITAL REPORTER IN PLANTS" CURRENT BIOLOGY, vol. 6, no. 3, 1 March 1996, pages 325-330, XP000571865 see the whole document	1,3-6,8, 12-17, 23,24, 28,29, 34,35, 38,40
X	PANG S. ET AL.: "An improved green fluorescent protein gene as a vital marker in plants" PLANT PHYSIOLOGY, vol. 112, no. 3, November 1996, pages 893-900, XP002064548 * see esp. pp. 896/97, p. 897 first paragraph *	1,3,5-8, 12-19, 23,24, 28,29, 34,35, 38,40

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 98/00359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9627675 A	12-09-1996	AU 4884396 A	23-09-1996